REMARKS

The Examiner rejected claims 1-16, 22-24, 28, 32, 33, and 37, while withdrawing claims 17-21, 25-27, 29-31, 34-36, and 38 from consideration. Claim 13 has been canceled herein without prejudice. In addition, claims 1, 8, 11, and 37 have been amended herein to indicate that the recited protein coding sequence is present in a Pir1, Pir2, Pir3 or Pir4 gene. Claims 1, 8, and 11 also have been amended to recite an enzyme as opposed to a peptide or a polypeptide. Claim 5 has been amended to recite an enzyme coding sequence that encodes a fucosyltransferase. Claim 24 has been amended to recite a *Saccharomyces cerevisiae* yeast cell as opposed to a microorganism. Applicants' specification fully supports these amendments. For example, on page 16, lines 23-26 disclose that a Pir gene can be a Pir1, Pir2, Pir3 or a Pir4 gene. On page 17, lines 17-21 disclose that a polypeptide of interest can be an enzyme such as a fucosyltransferase. On page 29, lines 30-32 disclose transformation of *S. cerevisiae*. Thus, no new matter has been added.

On page 29, line 2 of the specification cites YOKO-O *et al.*, FEBS, Vol.257, 1998. Applicants wish to point out that this citation is incorrect, and should have been YOKO-O *et al.* (*Euro. J. Biochem.*, 1998, 257: 630-637), a copy of which is attached for the Examiner's convenience.

In light of these amendments and the following remarks, Applicants respectfully request reconsideration and allowance of claims 1-12, 14-16, 22-24, 28, 32, 33, and 37.

Claim Objections

The Examiner objected to claim 3 as reciting a non-elected subject matter, since it recited SEQ ID NO:2. Claim 3 has been amended to remove the recitation of SEQ ID NO:2. Thus, this objection is moot.

The Examiner objected to claim 7 as being grammatically incorrect. Claim 7 has been amended to recite "said enzyme coding sequence" as opposed to "the peptide or a polypeptide coding sequence." Thus, this objection is moot.

Rejections under 35 U.S.C. § 112, second paragraph

The Examiner rejected claims 1-2, 5-16, 22-24, 28, 32-33, and 37 under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicants regard as their invention. Specifically, the Examiner stated that the phrases "a yeast Pir (protein internal repeat) cell wall protein coding sequence," "a Pir (protein internal repeat) motif coding sequence," and "a yeast cell wall protein coding sequence" in claims 1, 8, 11, and 37 are indefinite. The Examiner also stated that the term "GAP promoter" in claims 1, 8, 11, 14, 16, 22, and 37 is indefinite, and will be interpreted to include any promoter. In addition, the Examiner stated that the phrase "expression cassette....comprising an expression vector" in claims 9 and 10 is confusing, and that the phrases "host cell ...comprising a yeast cell wall," and "microorganism comprising a yeast cell wall" in claims 13 and 22 are unclear.

Applicants respectfully disagree. A person having ordinary skill in the art reading Applicants' specification would have understood the meaning of a yeast Pir cell wall protein coding sequence, a Pir motif coding sequence, and a yeast cell wall protein coding sequence. To further prosecution, however, claims 1, 8, 11, and 37 have been amended to recite "a protein coding sequence present in a Pir1, Pir2, Pir3 or Pir4 gene." A person having ordinary skill in the art reading Applicants' specification would have understood the meaning of a coding sequence that is present in a Pir1, Pir2, Pir3 or Pir4 gene.

Applicants respectfully submit that a person having ordinary skill in the art would have appreciated the meaning of GAP promoter domain at the time of filing. In fact, GAP promoters were known and used in the art at the time Applicants filed, as evidenced by, for example, the abstract of Sears *et al.*, 1998, *Yeast*, 14(8): 783-790. A copy of this abstract is attached for the Examiner's convenience. Applicants note that a "patent need not teach, and preferably omits, what is well known in the art." *Spectra Physics, Inc. v. Coherent, Inc.*, 827 F.2d 1524, 1534 (Fed. Cir. 1987).

Claim 9 has been amended as suggested by the Examiner to remove "comprising an expression vector." Thus, the rejection of claim 9 is moot. In addition, claim 13 has been

canceled, and claim 22 has been amended to remove the word "wall." Thus, these rejections are moot.

In light of the above, Applicants respectfully request the withdrawal of the rejection of claims 1-2, 5-12, 14-16, 22-24, 28, 32-33, and 37 under 35 U.S.C. § 112, second paragraph.

Rejections under 35 U.S.C. § 112, first paragraph

The Examiner rejected claims 1-16, 22-24, 28, 32-33, and 37 under 35 U.S.C. § 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to convey to one skilled in the relevant art that the inventors, at the time the application was filed, had possession of the claimed invention. Specifically, the Examiner stated that the claims lack written description, alleging that the genus of GAP promoter domains and the genus of yeast Pir cell wall protein coding sequences are not fully described by the instant specification. The Examiner further stated that the functional definition of these genera do not provide any structural information to aid in the identification of members of each genus. In addition, the Examiner appeared to characterize elements of the claimed invention as being essential or non-essential.

Applicants respectfully disagree with this rejection. A person having ordinary skill in the art reading Applicants' specification would have understood that Applicants invented the subject matter recited in the previously presented claims. To further prosecution, however, claims 1, 8, 11, and 37 have been amended to recite "an enzyme coding sequence" as opposed to "a peptide or a polypeptide coding sequence." In addition, claims 1, 8, 11, and 37 have been amended to recite "a protein coding sequence present in a Pir1, Pir2, Pir3 or Pir4 gene" as opposed to "a yeast Pir (protein internal protein) cell wall."

Applicants' specification adequately describes the subject matter of the presently amended claims. In fact, Applicants' specification describes a number of different enzymes such as a glycosyltransferase, a fucosyltransferase, a Lacto-N-fucopentaose, a galactosyltransferase, a glucosyltransferase, a mannosyltransferase, a galactosamyltransferase, a sialyltransferase, and an N-acetylglucosaminyltransferase. See, *e.g.*, page 17, lines 17-21 of Applicants' specification. In addition, Applicants' specification describes structural attributes of protein coding sequences

present in a Pir1, Pir2, Pir3, or Pir4 gene. For example, Applicants' specification provides the primary amino acid structure of Pir1 and Pir2. See, *e.g.*, SEQ ID NO:1 and SEQ ID NO:2. Applicants' specification also cites references that describe a number of different Pir genes. See, the section from page 16, line 23 to page 17, line 8. Moreover, the term "GAP promoter domain" is a descriptive term known in the art and used to distinguish GAP promoters from other promoters. For example, a person having ordinary skill in the art would not have confused a GAP promoter with other promoters. This is particularly true given that GAP promoters were known at the time Applicants filed, as evidenced by, for example, the abstract of *Sears et al.*, 1998, *Yeast*, 14(8): 783-790. Applicants note that "it is not required that the application describe the claim limitations in greater detail than the invention warrants. The description must be sufficiently clear that persons of skill in the art will recognize that the applicant made the invention having those limitations." *Martin v. Mayer*, 823 F.2d 500, 505 (Fed. Cir. 1987). Taken together, a person having ordinary skill in the art reading Applicants' specification and claims would have appreciated that Applicants have fully described Applicants' invention. Thus, the presently amended claims are adequately described.

With respect to the Examiner's apparent characterization of elements in the claims as essential or non-essential, Applicants are unaware of any case law establishing essentialness as a standard for determining compliance with the written description requirement. As explained above, a person having ordinary skill in the art reading Applicants' specification would have appreciated that Applicants invented the presently claimed subject matter.

In light of the above, Applicants respectfully request the withdrawal of the rejection of claims 1-12, 14-16, 22-24, 28, 32-33, and 37 under 35 U.S.C. § 112, first paragraph.

Rejections under 35 U.S.C. § 112, first paragraph

The Examiner rejected claims 1-16, 22-24, 28, 32-33, and 37 under 35 U.S.C. § 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it most nearly connected, to make and/or use the invention. Specifically, the Examiner maintained the lack of enablement rejection for the reasons previously of record. In addition, the Examiner questioned

the term "yeast Pir cell wall protein coding sequence" and Applicants' prior comments regarding this term. The Examiner also appeared to characterize elements of the claimed invention as being essential or non-essential.

Applicants respectfully disagree with this rejection. The test for enablement is whether one skilled in the art at the time Applicants filed the present application could make and use the claimed invention from the disclosures in the specification, coupled with the information known in the art, without "undue" experimentation. See, *e.g.*, MPEP § 2164.01. Factual considerations that can be weighed when determining whether "undue" experimentation would be required include: (1) the breadth of the claims, (2) the nature of the invention, (3) the state of the prior art, (4) the relative skill of those in the art, (5) the predictability or unpredictability of the art, (6) the amount of direction or guidance provided, (7) the presence or absence of working examples, and (8) the quantity of experimentation necessary. *See, In re Wands*, 858 F.2d 731, 737 (Fed. Cir. 1988). It is improper to conclude that a disclosure is not enabling based on an analysis of only one of the above factors while ignoring one or more of the others. In other words, all the evidence must be considered, and any conclusion of nonenablement must be based on the evidence as a whole. MPEP § 2164.01(a).

Applicants' specification enabled the previously presented claims. To further prosecution, however, claims 1, 8, 11, and 37 have been amended to recite "a protein coding sequence present in a Pir1, Pir2, Pir3 or Pir4 gene," as opposed to "a yeast Pir (protein internal protein) cell wall." In addition, claims 1, 8, 11, and 37 have been amended to recite "an enzyme coding sequence" as opposed to "a peptide or a polypeptide coding sequence."

A person having ordinary skill in the art reading Applicants' specification would have been able to follow the teachings provided throughout Applicants' specification to make and use the presently claimed invention without undue experimentation. For example, a person having ordinary skill in the art at the time Applicants filed would have been able to use standard cloning techniques to obtain (1) a protein coding sequence present in a Pir1, Pir2, Pir3, or Pir4 gene, (2) an enzyme coding sequence, and (3) a GAP promoter domain. This is particularly true given that Applicants' specification discloses the sequence for multiple Pir genes. In addition, Applicants specification discloses working examples that were used to obtain sequences present in Pir

genes. See, *e.g.*, the section extending from page 27, line 4 to page 28, line 10. Applicants' specification also discloses that many types of enzymes and GAP promoter can be used. Given the state of the art at the time Applicants filed, it is clear that a person having ordinary skill in the art would not require undue experimentation to obtain a sequence encoding an enzyme or a GAP promoter domain. For example, a person having ordinary skill in the art would have been able to follow Example 2 of Applicants' specification to obtain a sequence encoding a galactosyltransferase enzyme. See, page 28, lines 11-27.

Moreover, a person having ordinary skill in the art would have been able to use standard molecule cloning techniques to make a chimeric nucleic acid containing (1) a protein coding sequence present in a Pir1, Pir2, Pir3, or Pir4 gene, (2) an enzyme coding sequence, and (3) a GAP promoter domain. This is particularly true given that Applicants' specification discloses multiple working examples that disclose the construction of chimeric nucleic acids. Applicants' specification also teaches how to use the presently claimed chimeric nucleic acids. For example, Examples 3 and 4 disclose transforming yeast cells with a chimeric nucleic acid to express the encoded fusion polypeptide. See, the section extending from page 29, line 19 to page 30, line 27. Taken together, a person having ordinary skill in the art would have been able to make and use the presently claimed invention without undue experimentation. Thus, Applicants' specification fully enables the presently amended claims.

With respect to the Examiner's apparent characterization of elements in the claims as essential or non-essential, Applicants are unaware of any case law establishing essentialness as a standard for determining compliance with the enablement requirement of 35 U.S.C. § 112, first paragraph. As explained above, a person having ordinary skill in the art reading Applicants' specification would have appreciated that Applicants' specification fully enables the presently claimed subject matter.

In light of the above, Applicants respectfully request the withdrawal of the rejection of claims 1-12, 14-16, 22-24, 28, 32-33, and 37 under 35 U.S.C. § 112, first paragraph.

Rejections under 35 U.S.C. § 102

The Examiner rejected claims 1-2, 4, and 7 under 35 U.S.C. § 102(b) as being anticipated by Moukadiri *et al.* (*J. Bacteriol.*, 181:4741-4745 (1999)) for the reasons of record.

Applicants respectfully disagree. The Moukadiri *et al.* reference does not anticipate the previously presented claims. To further prosecution, however, claim 1 has been amended to recite "a protein coding sequence present in a Pir1, Pir2, Pir3 or Pir4 gene," as opposed to "a yeast Pir (protein internal protein) cell wall." In addition, claim 1 has been amended to recite "an enzyme coding sequence" as opposed to "a peptide or a polypeptide coding sequence." At no point does the Moukadiri *et al.* reference disclose a chimeric nucleic acid containing a GAP promoter domain. Thus, the presently claimed invention is not anticipated by the Moukadiri *et al.* reference.

In light of the above, Applicants respectfully request withdrawal of the rejection of claims 1-2, 4, and 7 under 35 U.S.C. § 102(b).

The Examiner also rejected claims 1-2, 4-16, 22-24, 28, 32-33, and 37 under 35 U.S.C. § 102(b) as being anticipated by Matilla *et al.* (*Glycobiol.*, 6:851-859 (1996)) for the reasons of record.

Applicants respectfully disagree. The Matilla *et al.* reference does not anticipate the previously presented claims. To further prosecution, however, claims 1, 8, 11, and 37 have been amended to recite "a protein coding sequence present in a Pir1, Pir2, Pir3 or Pir4 gene," as opposed to "a yeast Pir (protein internal protein) cell wall." In addition, claims 1, 8, 11, and 37 have been amended to recite "an enzyme coding sequence" as opposed to "a peptide or a polypeptide coding sequence." At no point does the Matilla *et al.* reference disclose a chimeric nucleic acid containing a GAP promoter domain. Thus, the presently claimed invention is not anticipated by the Matilla *et al.* reference.

In light of the above, Applicants respectfully request withdrawal of the rejection of claims 1-2, 4-12, 14-16, 22-24, 28, 32-33, and 37 under 35 U.S.C. § 102(b).

Rejections under 35 U.S.C. § 103

The Examiner rejected claims 1-16, 22-24, 28, 32-33, and 37 under 35 U.S.C. § 103(a) as being unpatentable over Matilla *et al.* (*Glycobiol.*, 6:851-859 (1996)) in view of Moukadiri *et al.* (*J. Bacteriol.*, 181:4741-4745 (1999)), Toh-e *et al.* (*Yeast*, 9:481-489 (1993)), and Mrsa (*Yeast*, 15:813-320 (1999)) for the reasons of record.

Applicants respectfully disagree. To establish a *prima facie* case of obviousness, the art must teach or suggest all limitations of the claim or claims at issue. See, *e.g.*, MPEP § 2143.03. Nowhere do the cited references, either alone or in combination, teach or suggest a chimeric nucleic acid containing a GAP promoter. In fact, none of the cited references teaches or suggests any nucleic acid containing a GAP promoter, let alone a chimeric nucleic acid containing (1) a protein coding sequence present in a Pir1, Pir2, Pir3, or Pir4 gene, (2) an enzyme coding sequence, and (3) a GAP promoter domain. Thus, the obviousness rejection is improper.

In light of the above, Applicants respectfully request withdrawal of the rejection of claims 1-12, 14-16, 22-24, 28, 32-33 and 37 under 35 U.S.C. § 103(a).

CONCLUSION

Applicants submit that claims 1-12, 14-16, 22-24, 28, 32, 33, and 37 are in condition for allowance, which action is requested. The Examiner is invited to call the undersigned agent at the telephone number below if such will advance prosecution of this application. The Commissioner is authorized to charge any fees or credit any overpayments to Deposit Account No. 06-1050.

Respectfully submitted,

Date: January 18, 2005

J. Patrick Finn III, Ph.D.
Reg. No. 44,109

Fish & Richardson P.C., P.A.
60 South Sixth Street, Suite 3300
Minneapolis, MN 55402
Telephone: (612) 335-5070
Fax: (612) 288-9696
60268347.doc

Eur. J. Biochem. 257, 630–637 (1998)

Differences in *in vivo* acceptor specificity of two galactosyltransferases, the *gmh3*$^+$ and *gma12*$^+$ gene products from *Schizosaccharomyces pombe*

Takehiko YOKO-O, Samir Kumar ROY and Yoshifumi JIGAMI

National Institute of Bioscience and Human Technology, Tsukuba, Ibaraki, Japan

(Received 11 May/13 July 1998) – EJB 98 0636/5

In the fission yeast *Schizosaccharomyces pombe*, *gmh1*$^+$, *gmh2*$^+$ and *gmh3*$^+$ genes encode α-1,2-galactosyltransferase homologues. In an *in vitro* galactosyltransferase assay, the *gmh3*$^+$ gene product showed galactose transfer activity toward α-methyl-D-mannoside as an acceptor. The disruption of *gma12*$^+$, the major galactosyltransferase gene [Chappell, T. G., Hajibagheri, M. A. N., Ayscough, K., Pierce, M. & Warren, G. (1994) *Mol. Biol. Cell 5*, 519–528], and of *gmh3*$^+$ in *S. pombe* caused decreases in the total remaining galactosyltransferase activity and cell surface galactose content. Disruption of *gma12*$^+$ and *gmh3*$^+$ also caused an increase in electrophoretic mobility of acid phosphatase, indicating their involvement in the galactosylation of cell surface glycoproteins. The *gmh3Δ gma12Δ* double mutant cells had more severe galactose-less phenotypes than single gene mutant cells. HPLC analysis of O-linked mannoprotein oligosaccharides from wild-type and disrupted strains revealed that the *gma12*$^+$ gene product is responsible for the galactosylation of O-linked oligosaccharide, whereas *gmh3*$^+$ has no involvement in the process. In contrast, both the *gmh3*$^+$ and *gma12*$^+$ gene products are involved in the galactosylation of the N-linked core oligosaccharide $Man_9GlcNAc_2$. From these results, it is evident that there are some functional differences between the enzymes in the process of galactosylation. It appears that the *gmh3*$^+$ gene product transfers galactose to N-linked oligosaccharide, while the *gma12*$^+$ gene product transfers galactose to both N-linked and O-linked oligosaccharides.

Keywords: fission yeast; galactosyltransferase; glycoprotein; oligosaccharide; *Schizosaccharomyces pombe*.

The fission yeast *Schizosaccharomyces pombe* is one of the most suitable model organisms to study cell cycle regulation [1], protein secretion and simultaneous post-translational modifications such as glycosylation [2–4]. In yeast, protein glycosylation, the most common post-translational modification, starts in the endoplasmic reticulum (ER) and continues through the Golgi compartments during the transport of glycoproteins to their final destination [5, 6].

Unlike the budding yeast *Saccharomyces cerevisiae*, the glycoproteins of *S. pombe* contain large amounts of D-galactose in addition to D-mannose [7] and have been called galactomannoproteins [8]. These galactomannoproteins contain both O-linked and N-linked carbohydrates, where galactose is attached to mannose or another galactose by an α-1,2-linkage [4]. To elucidate the physiological function of galactosylation in *S. pombe*, galactosylation-defective mutants were isolated [9–11]. The mutations showed little, if any, effect on vegetative cell growth. They were mapped to the genes encoding UDP-glucose-4-epimerase, the enzyme responsible for the interconversion between UDP-glucose and UDP-galactose [10], or the UDP-galactose transporter, which translocates UDP-galactose from the cytosol to the Golgi lumen [12].

Correspondence to Y. Jigami, National Institute of Bioscience and Human Technology, 1-1 Higashi, Tsukuba, Ibaraki, Japan 305-8566

Fax: +81 298 54 6220.

E-mail: jigami@nibh.go.jp

Abbreviations. ER, endoplasmic reticulum; Gma12p, *gma12*$^+$ gene product; Gmh1p, *gmh1*$^+$ gene product; Gmh2p, *gmh2*$^+$ gene product; Gmh3p, *gmh3*$^+$ gene product; GS I-B4, *Griffonia simplicifolia* lectin I-B4; PA, pyridylamino.

However, to elucidate the physiological function of cell surface galactosylation, characterisation of the galactosyltransferase that incorporates galactose into the oligosaccharide acceptors was necessary. A major galactosyltransferase was purified [13] and the corresponding gene was cloned and identified as *gma12*$^+$ [14]. The deletion strain of *gma12*$^+$ was viable, having a significantly reduced level of remaining galactosyltransferase activity. The presence of other galactosyltransferase activities was demonstrated through *in situ* lectin blotting and *in vitro* biochemical assays [14].

In this paper, we describe the characterisation of a novel galactosyltransferase encoded by *gmh3*$^+$ in combination with the functional analysis of the *gma12*$^+$ gene product (Gma12p). We also report on functional differences between both enzymes in galactose incorporation into N-linked and O-linked oligosaccharides of cell surface galactomannoproteins.

MATERIALS AND METHODS

Strains, media and genetic methods. The fission yeast strains used in this study are listed in Table 1. The media used for culture of the fission yeast cells were essentially those described by Moreno et al. [15]. YES–P medium, which contains a reduced amount of inorganic phosphate, was prepared as follows: 2.5 g Bacto-yeast extract (Difco) was dissolved in 400 ml H_2O. Then, 5 ml 1 M $MgSO_4$ and 5 ml 28% ammonia solution were added to precipitate the inorganic phosphate. The solution was stirred for 30 min and subsequently left at room temperature for 10 min. The precipitate was removed by filtration. After ad-

Table 1. *S. pombe* strains.

Name	Genotype	Source
JY741	*h⁻ ura4-D18 leu1 ade6-M216*	gift from M. Yamamoto
FTY1	*h⁻ ura4-D18 leu1 ade6-M216 gmh1Δ::ura4⁺*	this study
FTY2	*h⁻ ura4-D18 leu1 ade6-M216 gmh2Δ::ura4⁺*	this study
FTY3	*h⁻ ura4-D18 leu1 ade6-M216 gmh3Δ::ura4⁺*	this study
FTY4	*h⁻ ura4-D18 leu1 ade6-M216 gmh3Δ::LEU2*	this study
FTY5	*h⁻ ura4-D18 leu1 ade6-M216 gma12Δ::ura4⁺*	this study
FTY6	*h⁻ ura4-D18 leu1 ade6-M216 gmh3Δ::LEU2 gma12Δ::ura4⁺*	this study

justing the pH to 5.4 with HCl, 15 g glucose and 100 mg each of adenine sulfate, uracil, L-histidine hydrochloride, L-leucine and L(+)-lysine hydrochloride were added and the total volume was adjusted to 500 ml. Transformation of the fission yeast was performed by the lithium acetate method of Okazaki et al. [16]. The budding yeast strain used in this study was R16B (*MATa mnn1 leu2 ura3 trp1 ade8 his1* and/or *his3 lys2*) [17]. The media used for culture of the budding yeast cells were essentially those described by Sherman et al. [18]. Standard techniques for molecular cloning were adopted from those of Sambrook et al. [19].

***In vitro* amplification of *S. pombe* galactosyltransferase gene.** PCR was carried out essentially as described by Saiki et al. [20]. Based on two conserved amino acid sequences, NG(I/L)NAGS(F/I/L)(F/L)(F/I/V)RN and (D/Q)(D/E/K)GDL(A/C/V)(I/L)HFAGC, which are found in the *gma12⁺*, SPAC5H10.11 and SPAC5H10.13c gene products, two oligonucleotide primers were designed for PCR amplification. The sequences of the designed primers are as follows: 5′-cccccgaattcAAYGGIITIAAYGCIGGIWSIITIYTIITIMGIAA-3′ and 5′-cccccggatccCAICCIGCRAARTGIAIIIMIARRTCICCITYIT-3′, where capital letters indicate the coding region and the letter I indicates inosine, R indicates A/G, Y indicates C/T, W indicates A/T, S indicates C/G, and M indicates A/C mixture. PCR was carried out using 0.5 μM of these primers and 200 ng of genomic DNA of *S. pombe* strain 972*h⁻* as a template in the 20-μl reaction mixture. LA *Taq* polymerase (Takara) was used for the reaction. The reactions were performed in 30 cycles as follows: the samples were heated to 98°C for 20 s, cooled to 37°C for 1 min and heated to 72°C for 2 min.

Construction of plasmids. Construction of plasmid YEpUGAP-gma12⁺, which carries the *S. pombe gma12⁺* gene under the control of the *TDH3* promoter of *S. cerevisiae*, was described previously [17]. Plasmid YEp352GAP was constructed as follows. First, two plasmids were constructed. One was a modified plasmid of pKT10 [21], the *Hin*dIII site of which was replaced by a *Bam*HI site. The other was a modified plasmid of YEp352 [22], whose multiple cloning site was removed by *Pvu*II digestion and replaced by a *Bam*HI site. A 1.0-kb *Bam*HI fragment containing the *TDH3* promoter and terminator from the modified pKT10 was inserted into the *Bam*HI site of the modified YEp352 to construct YEp352GAP.

Plasmids pSK++gmh1⁺(S), pSK++gmh2⁺(S) and pSK++gmh3⁺(S) carry 1.0-kb *Eco*RI/*Xho*I fragments containing *gmh1⁺*, *gmh2⁺* and *gmh3⁺* open reading frames, respectively, amplified by PCR in pBluescript II SK+ (Stratagene). Plasmid YEpUGAP-gmh1⁺ was constructed as follows: the 0.6-kb *Eco*RI/*Xho*I fragment of pSK++gmh1⁺(S) was inserted into plasmid YEp352GAP to yield plasmid YEp352GAP+gmh1C. Subsequently, the 0.4-kb *Eco*RI fragment of pSK++gmh1⁺(S) was inserted into plasmid YEp352GAP+gmh1C to yield YEpUGAP-gmh1⁺. Plasmids YEpUGAP-gmh2⁺ and YEpUGAP-gmh3⁺ were constructed by inserting the 1.0-kb *Eco*RI/*Xho*I fragments of pSK++gmh2⁺(S) and pSK++gmh3⁺(S), respectively, into YEp352GAP.

Plasmid pSK++gmh1Δ::ura4⁺ was constructed as follows. Plasmid pSK++gmh1⁺(L) carries a 1.6-kb *Nru*I/*Xba*I fragment containing the *gmh1⁺* open reading frame amplified by PCR in pBluescript II SK+. The 0.7-kb *Hin*dIII fragment, located in the *gmh1⁺* open reading frame of pSK++gmh1⁺(L), was removed and a 1.8-kb *Hin*dIII fragment containing the selectable marker gene *ura4⁺* from pP(ura4⁺) (kindly provided by T. Toda, Imperial Cancer Research Fund, UK) was inserted to construct pSK++gmh1Δ::ura4⁺. pSK++gmh1Δ::ura4⁺ was digested with *Pvu*II to cut sites located on both sides of a multiple cloning site of pBluescript II SK+ and used for gene disruption.

Plasmid pSK++gmh2Δ::ura4⁺ was constructed as follows. Plasmid pSK++gmh2⁺(L) carries a 2.2-kb *Mlu*I/*Afl*II fragment containing the *gmh2⁺* open reading frame amplified by PCR in pBluescript II SK+. The 1.1-kb *Hpa*I/*Eco*RV fragment, located in the *gmh2⁺* open reading frame of pSK++gmh2⁺(L), was removed and a 1.8-kb *Hin*dIII fragment from pP(ura4⁺) was inserted to construct pSK++gmh2Δ::ura4⁺. pSK++gmh2Δ::ura4⁺ was digested with *Apa*I and *Sac*II to cut sites located at the multiple cloning site of pBluescript II SK+ and used for gene disruption.

Plasmid pSK++gmh3Δ::ura4⁺ was constructed as follows. Plasmid pSK++gmh3⁺(L) carries a 3.5-kb *Bgl*II/*Xho*I fragment containing the *gmh3⁺* open reading frame amplified by PCR in pBluescript II SK+. The 0.6-kb *Stu*I/*Eco*RV fragment, located in the *gmh3⁺* open reading frame of pSK++gmh3⁺(L), was removed and a 1.8-kb *Hin*dIII fragment from pP(ura4⁺) was inserted to construct pSK++gmh3Δ::ura4⁺. pSK++gmh3Δ::ura4⁺ was digested with *Pvu*II to cut sites located on both sides of a multiple cloning site of pBluescript II SK+, and used for gene disruption.

Plasmid pSK++gma12Δ::ura4⁺ was constructed as follows. Plasmid pSK++gma12⁺ carries a 1.1-kb *Eco*RI/*Xho*I fragment containing the *gma12⁺* open reading frame amplified by PCR in pBluescript II SK+. The 0.1-kb *Sal*I/*Bgl*II fragment, located in the *gma12⁺* open reading frame of pSK++gma12⁺, was removed and a 1.8-kb *Hin*dIII fragment from pP(ura4⁺) was inserted to construct pSK++gma12Δ::ura4⁺. pSK++gma12Δ::ura4⁺ was digested with *Pvu*II to cut sites located on both sides of a multiple cloning site of pBluescript II SK+ and used for gene disruption.

Plasmid pAT539, which was used for replacing *ura4⁺* with *S. cerevisiae LEU2*, was kindly provided by A. Toh-e (University of Tokyo) [23]. The *Hin*dIII fragment containing the *ura4Δ::LEU2* gene was excised from pAT539 and used for gene disruption.

Preparation of solubilised microsomal proteins. Solubilised enzymes from various cells were prepared as described previously [17]. Briefly, 10 g (wet mass) of freshly grown cells were washed with ice-cold water and resuspended in 10 ml TMS

buffer (20 mM Tris/HCl, 5 mM $MgCl_2$ and 0.25 M sucrose, pH 7.5). Cells were broken with 0.5-mm glass beads in a Bead Beater (B. Braun Biotech). Glass beads, unbroken cells and large cell debris were removed by centrifugation at 10000×*g* at 4°C. The resultant supernatant was centrifuged at 100000×*g* for 1 h at 4°C. The membrane pellet was resuspended in TMS buffer to a protein concentration of 25 mg/ml and microsomes were disrupted and solubilised by the addition of Triton X-100 to a final concentration of 2% (by vol.). After incubating on ice for 30 min, the mixture was centrifuged at 100000×*g* for 1 h. The supernatant was used as the source of solubilised enzyme for the galactosyltransferase assay.

Protein quantification. Protein concentration was measured by BCA reagent (Pierce) using BSA as a standard.

Galactosyltransferase assay using ^{3}H-labelled nucleotide sugar as a donor. The standard transferase assays were carried out according to Chappell and Warren [13]. The reaction mixture in a total volume of 50 µl contained 50–80 µg of solubilised protein in 100 mM Hepes/NaOH, pH 7.0, 1 mM $MnCl_2$ with 25 nmol UDP-[^{3}H]galactose (specific activity 40 nCi/nmol, after dilution of commercially available UDP-[^{3}H]galactose with unlabelled UDP-galactose) and 5 µmol of a given sugar acceptor. The reaction mixture contained 0.1–0.2% (by vol.) Triton X-100, depending on the detergent concentration in the enzyme fraction. After incubation at 30°C for 30 min, the reaction was terminated by adding 200 µl ice-cold water and loaded onto a 1.0-ml Dowex-1 (Cl^- form, 200–400 dry mesh) anion exchange column packed in a 3-ml syringe. The column was washed twice with 1.0 ml water and the combined eluents were mixed with 2.5 volumes of scintillation cocktail. The Dowex columns were regenerated by washing in 2.5 ml 5 M NaCl and 2.5 ml 0.1 M HCl and then 8.0 ml water. Under standard assay conditions the reaction velocity was linear up to 60 min and 200 µg solubilised protein per assay for both cases.

Galactosyltransferase assay by pyridylamino (PA)-labelled oligosaccharides as an acceptor. The galactosyltransferase assay was carried out in 30 µl reaction mixture containing 600 µg solubilised enzyme, 100 mM Hepes/NaOH, pH 7.2, 1 mM $MnCl_2$, 5 mM UDP-galactose, 500–1000 pmol $Man_9GlcNAc_2$–PA acceptor (Takara; depending on the acceptor used) and incubated at 37°C for 2 h. The reaction was terminated by adding 200 µl ice-cold water and the reaction mixture was filtered through an Ultrafree-MC membrane (10K cut; Millipore). The filtrate was freeze-dried and subjected to HPLC analysis.

Fluorescence staining. Cells (5×10^6) were collected, fixed in 70% ethanol and washed with GS buffer (100 mM Hepes/NaOH, 1 mM $MgCl_2$, 100 µM $CaCl_2$ and 100 µM $MnCl_2$, pH 7.0). They were then incubated in 50 µl of GS buffer containing 0.5 µl fluorescein-conjugated *Griffonia simplicifolia* lectin I B4 (GS I-B4; Vector Laboratories) at 4°C for 1 h. Cells were washed with GS buffer three times and resuspended in 2 ml GS buffer. After sonication, they were analysed on the FACSCalibur/Cell Quest system (Becton Dickinson).

Analysis of acid phosphatase. Acid phosphatase from the fission yeast was analysed as described by Huang and Snider [10] with some modifications. The cells were grown in 20 ml YES medium to an optical density at 660 nm of approximately 3. To induce production of acid phosphatase, 4×10^8 cells were centrifuged, resuspended in 20 ml of YES–P, and incubated at 30°C for 8 h. The cells (8×10^8) were then collected by centrifugation, washed once with 62.5 mM Tris/HCl, pH 6.8, and suspended in 240 µl ice-cold lysis buffer (62.5 mM Tris/HCl, 1 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 0.1 mM dithiothreitol and 10% glycerol, pH 6.8). Cell lysates were prepared by vigorously mixing the cell suspension ten times with 0.5-mm glass beads for 20 s at 4°C. After adding 120 µl lysis buffer, the lysates were recovered and centrifuged at 14000×*g* for 10 min, and the supernatants were recovered and centrifuged again at 14000×*g* for 20 min. Supernatants were recovered again, and 2- to 10-µl aliquots were mixed with 1/3 volume of 0.01% bromophenolblue, 15% glycerol and 62.5 mM Tris/HCl, pH 6.8. Samples were immediately subjected to electrophoresis on 6% native polyacrylamide gel. Electrophoresis buffer and the method for activity staining of acid phosphatase were adopted from those of Schweingruber et al. [24].

HPLC analysis of O-linked oligosaccharide. Total cell surface mannoproteins were isolated as described by Nakanishi-Shindo et al. [25]. After gel filtration on a NAP-10 column (Pharmacia), O-linked oligosaccharides were prepared by hydrazinolysis (Hydrazinolysis Reagent C; Honen) using Hydraclub S-204 (Honen) according to manufacturer's protocol. Crude oligosaccharides were filtered through an Ultrafree-MC membrane (10-K cut) and pyridylaminated using a pyridylamination kit (Takara) according to the manufacturer's protocol. The PA-labelled sample was resuspended in H_2O and applied to a boric acid column (Pierce), and O-linked oligosaccharides were eluted by 0.2 M sorbitol. The boric-acid-column-binding fraction was further analysed by Asahipak NH2P-50 (4.6 mm×250 mm; Showa Denko) at a flow rate of 1.0 ml/min with solvent A (acetonitrile) and solvent B (200 mM acetic acid/triethylamine, pH 7.3). After sample injection, the proportion of solvent B was increased linearly from 10% to 60% for 60 min. Each peak was collected and then subjected to either α-mannosidase (EC 3.2.1.24; from Jack bean; Seikagaku) or α-galactosidase (EC 3.2.1.22; from green coffee bean; Sigma) treatment according to manufacturers' protocols.

HPLC analysis of N-linked oligosaccharide. PA-labelled N-linked oligosaccharides were analysed by TSKgel Amide-80 (4.6 mm×250 mm; Tosoh) at a flow rate of 1.0 ml/min with solvent A (70% of acetonitrile and 30% of 200 mM acetic acid/triethylamine, pH 7.2) and solvent B (30% of acetonitrile and 70% of 200 mM acetic acid/triethylamine, pH 7.2). After sample injection, the proportion of solvent B was increased linearly from 10% to 50% for 50 min.

RESULTS

Identification of a novel galactosyltransferase. We have searched for genes homologous to *gma12*$^+$ in the GenBank nucleotide database and identified two *gma12*$^+$-related genes of *S. pombe*, SPAC5H10.11 and SPAC5H10.13c, cloned in the *S. pombe* chromosome I sequencing project (Barrell et al., direct submission; GenBank accession number Z49811). We have also searched for further homologues to obtain other novel galactosyltransferases by PCR. Among the 72 PCR products sequenced, 13 products had the same sequence. The amino acid sequence deduced from this common sequence is highly similar to the corresponding sequence of Gma12p, suggesting that the amplified fragment was derived from a novel galactosyltransferase. In the course of the above gene cloning, the sequence corresponding to the PCR product was deposited as a gene SPAC22E12.06c in the *S. pombe* chromosome I sequencing project (Barrell et al., direct submission; GenBank Accession Number Z70043). For convenience, the SPAC5H10.11, SPAC5H10.13c and SPAC22E12.06c genes are referred to as *gmh1*$^+$, *gmh2*$^+$ and *gmh3*$^+$, respectively (*gma12*$^+$ homologue).

***gmh3*$^+$ gene product has galactosyltransferase activity.** To the best of our knowledge, no galactosyltransferase is present in *S. cerevisiae*. To determine whether the *gmh1*$^+$, *gmh2*$^+$ and *gmh3*$^+$ gene products (Gmh1p, Gmh2p and Gmh3p, respectively)

Table 2. Expression of *S. pombe* α1,2-galactosyltransferase gene in *S. cerevisiae*. The microsomal fractions of *S. cerevisiae* R16B cells solubilised by Triton X-100 as described in Materials and Methods were used as the enzyme source. α-Methyl-D-mannoside (5 μmol/50 μl reaction mixture) was used as sugar acceptor.

Plasmid	Galactosyltransferase activity
	nmol · min^{-1} · mg protein^{-1}
None	0.3[a]
pKT10	0.3[a]
YEpUGAP-gmh1$^+$	0.3
YEpUGAP-gmh2$^+$	0.3
YEpUGAP-gmh3$^+$	1.0
YEpUGAP-gma12$^+$	10.6[a]

[a] Data published by Roy et al. [17].

Table 3. Sugar acceptor specificity of expressed α-1,2-galactosyltransferase. Values were expressed as the percent of the activity obtained with α-methyl-D-mannoside as an acceptor which was taken as 100%. One hundred per cent activity corresponds to 10.6 and 1.0 nmol · min^{-1} · mg protein^{-1} for Gma12p and Gmh3p, respectively.

Acceptor	Relative activity	
	Gmh3p	Gma12p
	%	
α-Methyl-D-mannoside	100.0	100.0[a]
α-Methyl-D-galactoside	5.1	5.0[a]
D-Mannose	30.9	32.0[a]
D-Galactose	5.0	5.2[a]
Mannobiose (α-1,2-linked)	72.0	42.0
Mannotriose (α-1,2-linked)	15.0	18.0[a]
N-Acetyl-D-glucosamine	5.0	5.0[a]
None	4.8	4.8[a]

[a] Data published by Roy et al. [17].

have galactosyltransferase activity, they were expressed in *S. cerevisiae* R16B cells under the control of the *TDH3* promoter. Solubilised microsomal fractions were prepared to measure the galactosyltransferase activity. Of these gene products, only Gmh3p showed galactosyltransferase activity toward α-methyl-D-mannoside as an acceptor molecule (Table 2). Under standard assay conditions, Gmh3p showed approximately 10% transferase activity compared to Gma12p.

K_m values of Gma12p and Gmh3p were almost the same (2.5 mM and 2.9 mM, respectively) for donor substrate UDP-galactose using 100 mM α-methyl-D-mannoside as an acceptor substrate. When acceptor concentration was increased from 100 mM to 400 mM, K_m of Gma12p remained unchanged (2.7 mM) while that of Gmh3p was reduced to 1.3 mM. The K_m for α-methyl-D-mannoside using 0.5 mM UDP-galactose is 20 mM for Gma12p and 370 mM for Gmh3p. These data suggest that Gmh3p is different from Gma12p in its kinetic parameter. The other two candidates, Gmh1p and Gmh2p, did not show any detectable galactosyltransferase activity toward α-methyl-D-mannoside, even in the presence of higher concentrations of acceptor and donor substrates (data not shown).

Sugar acceptor specificity of Gmh3p was examined (Table 3) and compared with the specificity of Gma12p [17]. The acceptor specificities of Gmh3p and Gma12p were similar in that Gmh3p preferred mannose residues to galactose residues; however, the relative activity of Gmh3p was higher than that of Gma12p toward α-1,2-linked mannobiose.

Gene disruption of *gmh* genes in *S. pombe*. To investigate the function of *gmh* genes, the chromosomal *gmh1*$^+$, *gmh2*$^+$ and *gmh3*$^+$ genes were disrupted. First, disruption plasmids were constructed which carry *gmh1Δ*::*ura4*$^+$, *gmh2Δ*::*ura4*$^+$ and *gmh3Δ*::*ura4*$^+$, where most of the *gmh* coding sequences were replaced by *ura4*$^+$ (Fig. 1). These plasmids were then introduced into the haploid fission yeast strain JY741 by replacement transformation. Uracil-independent transformants were isolated, and disruption of the genomic *gmh* genes was confirmed by PCR (data not shown). All the resultant cells grew as well as wild-type cells without any apparent phenotypic defect, indicating that *gmh* genes are not essential for vegetative growth.

To examine the effect of disrupting two functional galactosyltransferase genes, *gmh3Δ gma12Δ* double mutant cells were constructed. First, the *ura4*$^+$ gene of *gmh3Δ*::*ura4*$^+$ was replaced with the *LEU2* gene of *S. cerevisiae*, which complements the defect of *leu1* mutation of *S. pombe*. Then the *gma12*$^+$ gene of the resultant *gmh3Δ*::*LEU2* cells and of JY741 cells was dis-

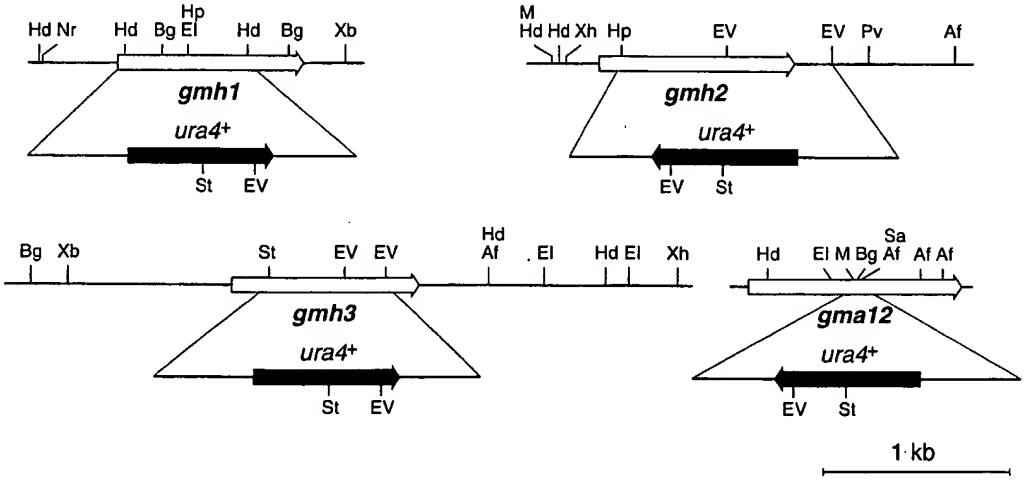

Fig. 1. Disruption of *gmh1*$^+$, *gmh2*$^+$, *gmh3*$^+$ and *gma12*$^+$ genes. The genes were replaced with *ura4*$^+$ selectable marker. Af, *Afl*II; Bg, *Bgl*II; EI, *Eco*RI; EV, *Eco*RV; Hd, *Hin*dIII; Hp, *Hpa*I; M, *Mlu*I; Nr, *Nru*I; Sa, *Sal*I; St, *Stu*I; Pv, *Pvu*II; Xb, *Xba*I; Xh, *Xho*I.

Table 4. Galactosyltransferase activity in *S. pombe*. The microsomal fractions solubilised by Triton X-100 as described in Materials and Methods were used as the enzyme source. α-methyl-D-mannoside (5 μmol/50 μl reaction mixture) was used as sugar acceptor. One hundred per cent corresponds to 2.8 nmol · min^{-1} · mg $protein^{-1}$.

Genotype of strain used for enzyme source	Galactosyltransferase activity
	%
WT	100
gmh1Δ::ura4⁺	100
gmh2Δ::ura4⁺	100
gmh3Δ::LEU2	77
gma12Δ::ura4⁺	25
gmh3Δ::LEU2 gma12Δ::ura4⁺	18

rupted with *ura4⁺* by replacement transformation (Fig. 1). Uracil-independent transformants were isolated, and disruption of the genomic *gma12⁺* gene was confirmed by PCR (data not shown). The *gmh3Δ::LEU2 gma12Δ::ura4⁺* cells showed no growth defect compared with wild-type and *gma12Δ::ura4⁺* cells, indicating that disruption of two galactosyltransferase genes has no effect on the normal vegetative growth.

Galactosyltransferase activity of *gmhΔ* cells. Previous results suggest that Gma12p is not the sole galactosyltransferase in *S. pombe* but rather contributes only part of the total galactosyltransferase activity [14]. Therefore, the remaining galactosyltransferase activity in the deletion strains was examined using α-methyl-D-mannoside as an acceptor under standard assay conditions (Table 4). Solubilised microsomal fractions from *gmh1Δ* or *gmh2Δ* strains had the same galactosyltransferase activity as that of wild-type cells, suggesting that neither Gmh1p nor Gmh2p contributes to the transferase activity, at least when α-methyl-D-mannoside is used as an acceptor. However, in *gmh3Δ* and *gma12Δ* strains galactosyltransferase activity was reduced to 77% and 25%, respectively, relative to that of wild-type cells. The solubilised microsomal fraction from *gmh3Δ gma12Δ* double mutant cells contained less activity than *gmh3Δ* or *gma12Δ* single mutant cells, proving that the effect of gene disruption is additive.

Content of galactose residues in *gma12Δ* and *gmh3Δ* cells. The cell surface of wild-type, *gmh3Δ* and *gma12Δ* single mutants and *gmh3Δ gma12Δ* double mutants was labelled with fluorescein-conjugated GS I-B4 lectin, which specifically recognises α-linked galactose. Labeled cells were subjected to flow cytometric analysis (Fig. 2). Both *gmh3Δ* and *gma12Δ* cells had less galactose in their cell surfaces than wild-type cells. Moreover, the galactose content in the cell surface of *gmh3Δ gma12Δ* double mutant cells was lower than that of single mutants, suggesting an additive effect on cell surface galactosylation by the disruption of *gmh3⁺* and *gma12⁺* genes.

To confirm the effect of gene disruption on protein galactosylation, the electrophoretic mobility of acid phosphatase, a typical glycoprotein in *S. pombe*, was investigated. Cell extracts from wild-type, *gma12Δ* and *gmh3Δ* single mutants and *gmh3Δ gma12Δ* double mutants were subjected to native polyacrylamide gel electrophoresis, and the position of acid phosphatase was detected by activity staining (Fig. 3). Both *gmh3Δ* and *gma12Δ* cells produced acid phosphatase with an increased electrophoretic mobility. The electrophoretic mobility of acid phosphatase from *gmh3Δ gma12Δ* double mutant cells was higher than that from single mutant cells. After digestion with endoglycosidase H, acid phosphatase from all strains exhibited the same mobility, suggesting that the difference in electrophoretic mobility was due to the difference in the extent of galactosylation of N-linked oligosaccharide.

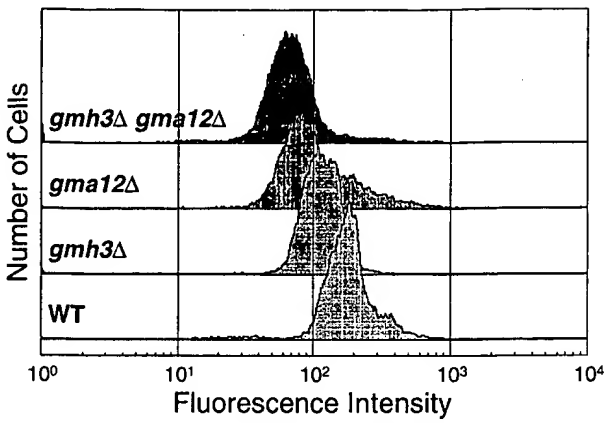

Fig. 2. Flow cytometric analysis of wild-type cells, *gmh3Δ::LEU2* and *gma12Δ::ura4⁺* single mutants and *gmh3Δ::LEU2 gma12Δ::ura4⁺* double mutants. Galactose residues on the cell surface were labelled with fluorescein-conjugated GSI-B4 lectin and the fluorescence intensity of each strain was measured by flow cytometry. One representative among five independent experiments, which yielded similar results, is shown.

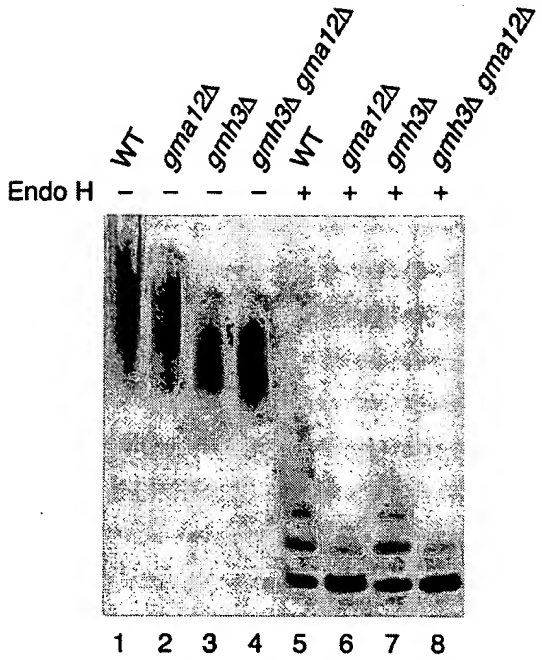

Fig. 3. Activity staining of acid phosphatase. Lysates from wild-type cells, *gma12Δ::ura4⁺* and *gmh3Δ::LEU2* single mutants and *gmh3Δ::LEU2 gma12Δ::ura4⁻* double mutants induced for acid phosphatase expression were subjected to electrophoresis on a 6% native gel and stained for acid phosphatase activity as described in Materials and Methods. Lanes 1–4, untreated lysates; lanes 5–8, lysates treated with endoglycosidase H.

Analysis of O-linked oligosaccharides in *gmh3Δ* and *gma12Δ* cells. The results of flow cytometric analysis and activity stain-

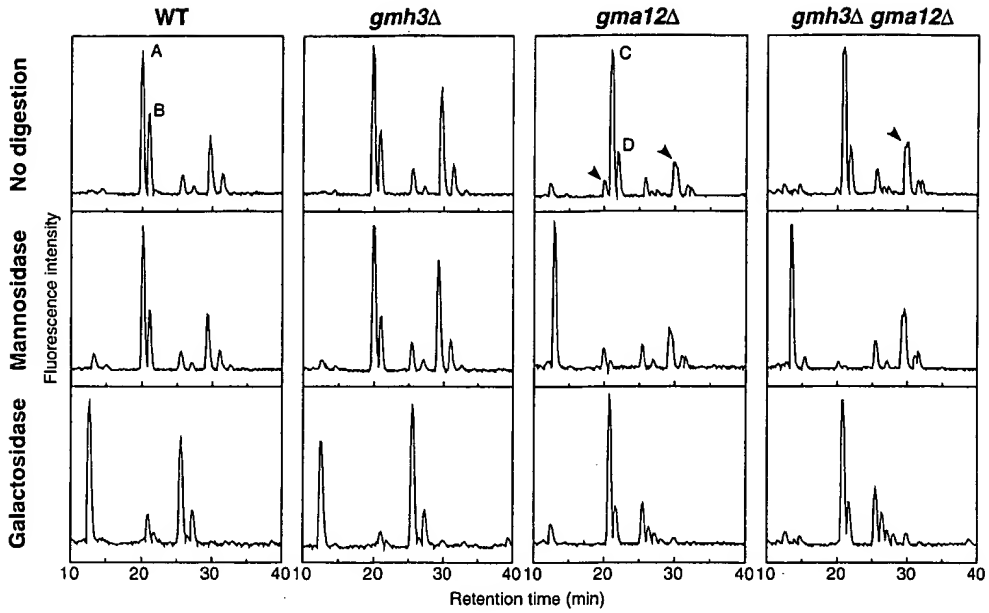

Fig. 4. HPLC profiles of O-linked oligosaccharides from galactomannoproteins. Galactomannoproteins were prepared from wild-type cells, *gmh3Δ::LEU2* and *gma12Δ::ura4*$^+$ single mutants and *gmh3Δ::LEU2 gma12Δ::ura4*$^+$ mutants. O-linked oligosaccharides were released by hydrazinolysis, pyridylaminated and analysed by HPLC using Asahipak NH2P-50. Oligosaccharides were digested with either Jack bean α-mannosidase (middle panels) or coffee bean α-galactosidase (lower panels) or were undigested. Peaks A, B, C and D, which correspond to disaccharides, were used for further analysis. Arrowheads indicate the peaks that disappeared after α-galactosidase digestion.

ing of acid phosphatase suggest some differences in galactomannan oligosaccharide structures between *gmh3Δ* and *gma12Δ* cells. To examine precisely the difference in the galactomannan structures of both cells, O-linked oligosaccharides were released from cell surface galactomannoproteins, pyridylaminated and analysed by HPLC.

HPLC profiles of O-linked oligosaccharides from each strain are shown in Fig. 4. These were classified into two groups: one from wild-type and *gmh3Δ* cells, and the other from the single mutant *gma12Δ* and the double mutant *gmh3Δ gma12Δ*. The disruption of the *gmh3*$^+$ gene had no effect on the HPLC profile of O-linked oligosaccharides, indicating that Gmh3p is not involved in the galactose incorporation into O-linked oligosaccharides.

To investigate the structure of O-linked oligosaccharides, pyridylaminated oligosaccharides were digested by Jack bean α-mannosidase (Fig. 4, middle panels) or coffee bean α-galactosidase (Fig. 4, lower panels). The HPLC profiles of the oligosaccharides from wild-type and *gmh3Δ* cells remained unchanged after α-mannosidase digestion but dramatically changed after α-galactosidase digestion, indicating the presence of galactose residues at most of the terminal positions. However, the HPLC profiles of the O-linked oligosaccharides from the *gma12Δ* single mutant and the *gmh3Δ gma12Δ* double mutant were dramatically changed by α-mannosidase digestion, indicating that the oligosaccharides from these mutants contain mannose residues in their terminal position. After α-galactosidase digestion, the peaks indicated by arrowheads in Fig. 4 disappeared (compare upper and lower panels), indicating that these mutants still contained some galactose residues in their terminal positions.

In order to analyse further the structure of O-linked oligosaccharides, major disaccharides in wild-type and *gma12Δ* cells, corresponding to peaks A, B, C and D in Fig. 4, were collected and digested by either α-mannosidase or α-galactosidase (data not shown). The position of peak A remained unchanged after the α-mannosidase digestion, but shifted to the position of monosaccharide after the α-galactosidase digestion, indicating that peak A consists of Gal-Man-PA. Peak B was separated into two peaks corresponding to monosaccharides and disaccharides after the α-mannosidase and the α-galactosidase digestion, showing that peak B contains Gal-Man-PA and Man-Man-PA. Peaks C and D were shifted to the position of monosaccharides only after the α-mannosidase digestion, indicating the structure of Man-Man-PA for peaks C and D. These results demonstrate that Gma12p is mainly involved in galactosylation at the second position (Gal-Man-*O*) and that, when the *gma12*$^+$ gene is disrupted, mannosyltransferase(s) transfers mannose instead of galactose to the second position (Man-Man-*O*).

Involvement of Gmh3p and Gma12p in galactosylation at the N-linked core oligosaccharide. The results of activity staining of acid phosphatase suggest that Gmh3p and Gma12p are involved in the galactosylation of N-linked oligosaccharides (Fig. 3). To obtain more information on the function of these galactosyltransferases in N-glycosylation, we assayed the galactosyltransferase activity of the solubilised microsomal fractions from wild-type, *gmh3Δ* and *gma12Δ* single mutant and *gmh3Δ gma12Δ* double mutant cells toward the pyridylaminated ER core oligosaccharide acceptor, $Man_9GlcNAc_2$-PA, by using UDP-galactose as a donor (Fig. 5). In the reaction using a membrane fraction from wild-type cells, peaks corresponding to $Gal Man_9GlcNAc_2$-PA and $Gal_2Man_9GlcNAc_2$-PA (peaks b and c in Fig. 5, respectively) were detected by HPLC, indicating the galactose transfer to $Man_9GlcNAc_2$. The activity was dependent on the presence of UDP-galactose and enzyme sources (data not shown). Approximately 22% of $Man_9GlcNAc_2$-PA was converted to $GalMan_9GlcNAc_2$-PA and 3% to $Gal_2Man_9GlcNAc_2$-PA. However, in the case of the *gmh3Δ* cells, 19% of $Man_9GlcNAc_2$-PA was converted to $GalMan_9GlcNAc_2$-PA and 2% to $Gal_2Man_9GlcNAc_2$-PA. In the case of the *gma12Δ* cells, 23% of

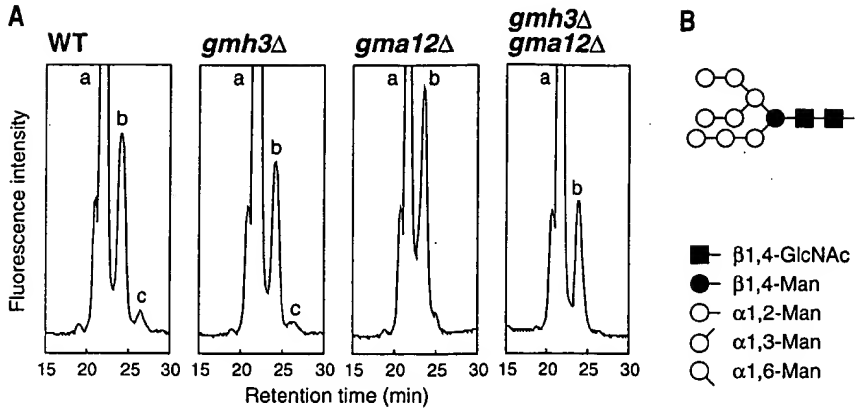

Fig. 5. Galactosyltransferase activity toward PA-$Man_9GlcNAc_2$. (A) The solubilised microsomal membrane fractions from wild-type cells, *gmh3Δ::LEU2* and *gma12Δ::ura4*$^+$ single mutants and *gmh3Δ::LEU2 gma12Δ::ura4*$^+$ double mutants were used as enzyme sources. After the enzyme reaction, the products were analysed by HPLC using TSKgel Amide-80. Peak a, $Man_9GlcNAc_2$-PA; peak b, $GalMan_9GlcNAc_2$-PA; peak c, $Gal_2Man_9GlcNAc_2$-PA. (B) Structure of $Man_9GlcNAc_2$ used as the acceptor.

$Man_9GlcNAc_2$-PA was converted to $GalMan_9GlcNAc_2$-PA, but none was converted to $Gal_2Man_9GlcNAc_2$-PA. Moreover, only 14% of $Man_9GlcNAc_2$-PA was converted to $GalMan_9GlcNAc_2$-PA in the *gmh3Δ gma12Δ* cells, indicating the synergistic effect of the double mutation on galactosylation. These results show that both Gmh3p and Gma12p contributed to galactose incorporation into an N-linked core oligosaccharide.

DISCUSSION

Properties of the Gmh3p galactosyltransferase. We have identified the galactosyltransferase gene, *gmh3*$^+$ which encodes Gmh3p. Gmh3p and Gma12p have different kinetic parameters: Gma12p exhibits a higher activity toward α-methyl-D-mannoside than does Gmh3p (Table 2), consistent with the finding that Gma12p is a major galactosyltransferase in *S. pombe* [13, 14]. Both enzymes have a K_m value in the millimolar range for UDP-galactose, in contrast to the K_m values of other glycosyltransferases, which register in the micromolar range for donors [26]. Both enzymes have a preference for a mannose residue over a galactose residue (Table 3). Judging from the acceptor substrate specificity, it is conceivable that Gmh3p and Gma12p are galactosyltransferases that transfer galactose to mannose residues but not to galactose residues.

Although galactose transfer activity was not detected for Gmh1p or Gmh2p under the assay conditions used here (Table 2), the possibility that Gmh1p and Gmh2p may be galactosyltransferases which do not recognise α-methyl-D-mannoside as an acceptor should not be excluded. Since the presence of a galactose residue that is attached to another galactose has been reported [4], it is possible that *gmh1*$^+$ and *gmh2*$^+$ encode the enzymes which transfer galactose to other galactose residues.

Phenotypes of *gmh3Δ* and *gma12Δ* cells. The *gmh3Δ gma12Δ* double mutant cells have no apparent growth defect, suggesting the following two possibilities: (1) double mutant cells can grow normally due to the remaining galactosyltransferase activity, or (2) the cells do not require galactose residues for their vegetative growth. Since it has been reported that the disruption of the gene encoding the UDP-galactose transporter causes a lack of galactose residues in cell surface glycoproteins without any defect in vegetative cell growth [12], the latter possibility seems more likely.

The disruption of *gmh3*$^+$ and *gma12*$^+$ caused a reduction in galactosylation at the cell surface (Fig. 2), proving the involvement of both Gmh3p and Gma12p in the incorporation of galactose into cell surface galactomannoproteins. The results of galactosyltransferase assays, flow cytometric analyses and activity staining of acid phosphatase show that the effects of the *gmh3Δ gma12Δ* double mutation accentuate the reduction of galactose incorporation (Table 4, Figs 2 and 3), suggesting that Gmh3p and Gma12p have some independent functions.

Galactosyltransferase activity of Gmh3p and Gma12p toward N-linked and O-linked oligosaccharides. The electrophoretic mobility of acid phosphatase suggests that both *gmh3Δ* and *gma12Δ* cells are defective in N-glycosylation (Fig. 3). Since *S. pombe* cells have no ER Man_9-α-1,2-mannosidase activity, the smallest ER core oligosaccharide in *S. pombe* is $Man_9GlcNAc_2$, not the $Man_8GlcNAc_2$ that is present in *S. cerevisiae* [3, 4]. Both *gmh3Δ* and *gma12Δ* cells have reduced galactosyltransferase activity toward a $Man_9GlcNAc_2$ N-linked core oligosaccharide (Fig. 5), indicating that Gmh3p and Gma12p are involved in the galactosylation of $Man_9GlcNAc_2$. We think that $Man_9GlcNAc_2$ is one of the natural acceptors for Gmh3p and Gma12p galactosyltransferases.

The position of galactose incorporation into $Man_9GlcNAc_2$ by Gmh3p and Gma12p remains to be elucidated. We digested the enzymatic reaction products $GalMan_9GlcNAc_2$-PA and Gal_2-$Man_9GlcNAc_2$-PA with coffee bean α-galactosidase and found that approximately 60% of incorporated galactose residues were resistant to α-galactosidase digestion (data not shown). Ballou et al. [4] showed the structure where the galactose residue is attached to the α-1,2-linked mannose residues in an N-linked core oligosaccharide. They reported that some of the galactose was rapidly released from the oligosaccharide by digestion with coffee bean α-galactosidase but some was released more slowly. Furthermore, resistance of α-linked galactose to α-galactosidases has been described [27]. Since our results are consistent with the reported observations, it is likely that Gmh3p and Gma12p transfer galactose to the α-1,2-linked mannose position in the N-linked core oligosaccharide, $Man_9GlcNAc_2$. In addition, it is also possible that they are involved in the galactosylation of the outer chain portion.

A HPLC analysis of O-linked oligosaccharides has revealed that Gma12p, but not Gmh3p, is responsible for the galactosylation of O-linked sugar chains (Fig. 4). O-linked oligosaccha-

rides from wild-type cells contain a large amount of galactosylmannose disaccharide (Fig. 4), as reported by Ballou et al. [4]. The HPLC profile of O-linked oligosaccharides in *gma12Δ* cells is different from that in wild-type cells in that a larger amount of mannobiose is accumulated in the former than in the latter (Fig. 4). This result indicates that Gma12p mainly transfers galactose to the O-linked mannose monomer and, when Gma12p is absent, mannosyltransferase(s) incorporates mannose into the O-linked mannose monomer. Therefore, it is conceivable that another natural acceptor for Gma12p galactosyltransferase is O-linked mannose. It was also observed that the heterologously produced Gma12p transferred galactose to the O-linked mannose monomer in *S. cerevisiae* cells (Kainuma, M., unpublished results). In wild-type *S. pombe* cells, it is likely that Gma12p and other mannosyltransferases function competitively in the second sugar transfer to the O-linked sugar chain.

The presence of galactose residues in N-linked oligosaccharides [3, 4] and O-linked oligosaccharides [4] has already been reported. Although the galactosyltransferase gene has been cloned as *gma12*$^+$ [14], it was unknown which oligosaccharide Gma12p transfers galactose to. This paper has dealt with the *in vivo* function of galactosyltransferases, demonstrating functional differences in acceptor specificity of Gmh3p and Gma12p. Gma12p is involved in the incorporation of galactose into both N-linked and O-linked oligosaccharides and plays a major role in the incorporation of galactose at the second position of O-linked oligosaccharide. Gmh3p is involved in the incorporation of galactose into N-linked core oligosaccharides. Further studies with the *gmh3Δ gma12Δ* double mutant cells will enable us to analyse the properties of other galactosyltransferase(s), which could be responsible for the addition of galactose to other galactose residues.

We are grateful to Dr M. Yamamoto (University of Tokyo), Dr T. Toda (Imperial Cancer Research Fund, UK) and Dr A. Toh-e (University of Tokyo) for providing *S. pombe* strains and plasmids. This work was supported in part by a grant-in-aid of the Research and Development Project of Basic Technologies for Future Industries from the Ministry of International Trade and Industry of Japan.

REFERENCES

1. Nurse, P. (1994) Ordering S phase and M phase in the cell cycle, *Cell 79*, 547–550.
2. Schwaninger, R., Dumermuth, E. & Schweingruber, M.E. (1990) Effects of seven different mutations in the *pho1* gene on enzymatic activity, glycosylation and secretion of acid phosphatase in *Schizosaccharomyces pombe*, *Mol. Gen. Genet. 221*, 403–410.
3. Ziegler, F. D., Gemmill, T. R. & Trimble, R. B. (1994) Glycoprotein synthesis in yeast. Early events in *N*-linked oligosaccharide processing in *Schizosaccharomyces pombe*, *J. Biol. Chem. 269*, 12527–12535.
4. Ballou, C. E., Ballou, L. & Ball, G. (1994) *Schizosaccharomyces pombe* glycosylation mutant with altered cell surface properties, *Proc. Natl Acad. Sci. USA 91*, 9327–9331.
5. Herscovics, A. & Orlean, P. (1993) Glycoprotein biosynthesis in yeast, *FASEB J. 7*, 540–550.
6. Lehle, L. & Tanner, W. (1995) Protein glycosylation in yeast, in *Glycoproteins* (Montreuil, J., Schachter, H. & Vliegenthart, J. F. G., eds) pp. 475–509, Elsevier Science, Amsterdam.
7. Bush, D. A., Horisberger, M., Horman, I. & Wursch, P. (1974) The wall structure of *Schizosaccharomyces pombe*, *J. Gen. Microbiol. 81*, 199–206.
8. Walther, P., Müller, M. & Schweingruber, M. E. (1984) The ultrastructure of the cell surface and plasma membrane of exponential and stationary phase cells of *Schizosaccharomyces pombe*, grown in different media, *Arch. Microbiol. 137*, 128–134.
9. Ballou, L. & Ballou, C. (1995) *Schizosaccharomyces pombe* mutants that are defective in glycoprotein galactosylation, *Proc. Natl Acad. Sci. USA 92*, 2790–2794.
10. Huang, K. M. & Snider, M. D. (1995) Isolation of protein glycosylation mutants in the fission yeast *Schizosaccharomyces pombe*, *Mol. Biol. Cell 6*, 485–496.
11. Takegawa, K., Tanaka, N., Tabuchi, M. & Iwahara, S. (1996) Isolation and characterization of a glycosylation mutant from *Schizosaccharomyces pombe*, *Biosci. Biotech. Biochem. 60*, 1156–1159.
12. Tabuchi, M., Tanaka, N., Iwahara, S. & Takegawa, K. (1997) The *Schizosaccharomyces pombe gms1*$^+$ gene encodes a UDP-galactose transporter homologue required for protein galactosylation, *Biochem. Biophys. Res. Commun. 232*, 121–125.
13. Chappell, T. G. & Warren, G. (1989) A galactosyltransferase from the fission yeast *Schizosaccharomyces pombe*, *J. Cell Biol.* 109, 2693–2702.
14. Chappell, T. G., Hajibagheri, M. A. N., Ayscough, K., Pierce, M. & Warren, G. (1994) Localization of an α1,2 galactosyltransferase activity to the Golgi apparatus of *Schizosaccharomyces pombe*, *Mol. Biol. Cell 5*, 519–528.
15. Moreno, S., Klar, A. & Nurse, P. (1991) Molecular genetic analysis of fission yeast *Schizosaccharomyces pombe*, *Methods Enzymol. 194*, 795–823.
16. Okazaki, K., Okazaki, N., Kume, K., Jinno, S., Tanaka, K. & Okayama, H. (1990) High-frequency transformation method and library transducing vectors for cloning mammalian cDNAs by *trans*-complementation of *Schizosaccharomyces pombe*, *Nucleic Acids Res. 18*, 6485–6489.
17. Roy, S. K., Yoko-o, T., Ikenaga, H. & Jigami, Y. (1998) Functional evidence for UDP-galactose transporter in *Saccharomyces cerevisiae* through the *in vivo* galactosylation and *in vitro* transport assay, *J. Biol. Chem. 273*, 2583–2590.
18. Sherman, F., Fink, G. R. & Hicks, J. B. (1986) *Laboratory course manual for methods in yeast genetics*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
19. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular cloning: a laboratory manual*, 2nd edn, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
20. Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B. & Erlich, H. A. (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase, *Science 239*, 487–491.
21. Tanaka, K., Nakafuku, M., Tamanoi, F., Kaziro, Y., Matsumoto, K. & Toh-e, A. (1990) *IRA2*, a second gene of *Saccharomyces cerevisiae* that encodes a protein with a domain homologous to mammalian *ras* GTPase-activating protein, *Mol. Cell. Biol. 10*, 4303–4313.
22. Hill, J. E., Myers, A. M., Koerner, T. J. & Tzagoloff, A. (1986) Yeast/*E. coli* shuttle vectors with multiple unique restriction sites, *Yeast 2*, 163–167.
23. Toh-e, A. (1995) Construction of a marker gene cassette which is repeatedly usable for gene disruption in yeast, *Curr. Genet. 27*, 293–297.
24. Schweingruber, A.-M., Schoenholzer, F., Keller, L., Schwaninger, R., Trachsel, H. & Schweingruber, M. E. (1986) Glycosylation and secretion of acid phosphatase in *Schizosaccharomyces pombe*, *Eur. J. Biochem. 158*, 133–140.
25. Nakanishi-Shindo, Y., Nakayama, K., Tanaka, A., Toda, Y. & Jigami, Y. (1993) Structure of the *N*-linked oligosaccharides that show the complete loss of α-1,6-polymannose outer chain from *och1*, *och1 mnn1*, and *och1 mnn1 alg3* mutants of *Saccharomyces cerevisiae*, *J. Biol. Chem. 268*, 26338–26345.
26. Hayes, B. K., Freeze, H. H. & Varki, A. (1993) Biosynthesis of oligosaccharides in intact Golgi preparations from rat liver, *J. Biol. Chem. 268*, 16139–16154.
27. Li, Y.-T. & Li, S.-C. (1972) α-Galactosidase from figs, *Methods Enzymol. 28*, 714–720.

1: Yeast. 1998 Jun 15;14(8):783-90.

A versatile set of vectors for constitutive and regulated gene expression in Pichia pastoris.

Sears IB, O'Connor J, Rossanese OW, Glick BS.

Department of Molecular Genetics and Cell Biology, University of Chicago, 60637, USA.

The budding yeast Pichia pastoris is an attractive system for exploring certai questions in cell biology, but experimental use of this organism has been limited by a lack of convenient expression vectors. Here we describe a set of compact vectors that should allow for the expression of a wide range of endogenous or foreign genes in P. pastoris. A gene of interest is inserted into modified pUC19 polylinker; targeted integration into the genome then result in stable and uniform expression of this gene. The utility of these vectors wa illustrated by expressing the bacterial beta-glucuronidase (GUS) gene. Constitutive GUS expression was obtained with the strong GAP promoter or the moderate YPT1 promoter. The regulatable AOX1 promoter yielded very strong GUS expression in methanol-grown cells, negligible expression in glucose-grown cells, and intermediate expression in mannitol-grown cells. GenBank Accession Numbers are: pIB1, AF027958; pIB2, AF0279959; pIB AF027960; pIB4, AF027961.

PMID: 9675822 [PubMed - indexed for MEDLINE]